EFFECTS OF SEED INOCULATION WITH *Azospirillum brasilense* AND NITROGEN DOSE ON OIL CONTENT OF CORN GRAINS

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ABSTRACT

Due to its high economical importance and the need of high amounts of nitrogen fertilizer, the corn crop demands further and deeper studies on biological nitrogen fixation trough bacterium *Azospirillum brasilense.* Therefore, this study aimed at assessing influence of inoculation of three different genotypes (Orion, A1 Bandeirante and AG-1051) with such bacterium in association with cover application of five different nitrogen doses on percentage oil content in the grains. Data were subjected to ANOVA and means compared by Tukey test at 5%. The inoculation of *A. brasilense*, with or without nitrogen fertilization, has promoted oil content increase in the grains. Orion genotype presented the greatest increase with the bacterium inoculation, as well as it was the most productive at the lowest nitrogen dosages assessed. High temperatures favored the increase in the oil content in the grains.

Keywords: *Zea mays*, inoculation, biological fixation, oil

EFEITOS DA INOCULAÇÃO COM A*zospirillum brasilense* E DOSE DE NITROGÊNIO SOBRE O TEOR DE ÓLEO DE MILHO

RESUMO

Devido à sua grande importância econômica e necessidade de fertilizantes nitrogenados, a cultura do milho exige estudos mais aprofundados sobre a fixação biológica do nitrogênio pela bactéria *Azospirillum brasilense*. Portanto, este estudo teve como objetivo avaliar a influência da inoculação de três diferentes genótipos (Orion, A1 Bandeirante e AG-1051) com essa bactéria em associação com a aplicação de cinco diferentes doses de nitrogênio no teor percentual de óleo nos grãos. Os dados foram submetidos à ANOVA e as médias comparadas pelo teste de Tukey a 5%. A inoculação de *A.*

brasilense, com ou sem adubação nitrogenada, promoveu aumento do teor de óleo nos grãos. O genótipo Orion apresentou o maior aumento com a inoculação da bactéria, bem como o mais produtivo nas menores dosagens de nitrogênio avaliadas. As temperaturas altas favorecem ou aumentam o teor de óleo nos grãos.

Palavras-chave: *Zea mays*, inoculação, fixação biológica, óleo

INTRODUCTION

Besides being an excellent low cost food source, and its cultivation viability on both large and small scale, the corn (*Zea mays* L.) is a plant grown in all continents and its main economic importance is due to its high productivity, to its high nutritional value, and to its ample forms of use as human and animal food source, as well as biofuel (GALVÃO, 2015).

The corn grain contains, on average circa 4% oil, but there are records of corn cultivars, in which seeds exceed 5% in oil content; besides, corn oil, which is extracted from the embryo of the seeds, contains unsaturated fatty acids on its composition (MENEGALDO, 2011).

According to Santos et al. (2016), the mean values of the oil content in grains of the UFT 8 and UFT 3 corn genotypes, obtained in an experiment where high, medium and low N dosages were applied, at two different sowing times, were found oil contents varying from 3.5 to 6.6% in the seeds, respectively; what corroborates the oil contents found in corn seeds reported in the literature.

The largest corn producers in the world are the United States, China and Brazil, with 345.5, 224.6 and 81 million tons, respectively, what together accounts for approximately 70% of world production, i.e. around 651.1 million tons (USDA, 2016).

In Brazil, corn is the second most cultivated grain, occupying a cropped area of 15,754.7 million hectares (CONAB, 2016) with an estimated production, for the 2016/2017 crop season, of 82 million tons (USDA, 2016). However, despite the importance of corn crop in the Brazilian economy, the mean productivity of the corn crop has not yet surpassed 4,389 kg ha^{-1} (CONAB, 2016).

In the state of Tocantins, the low corn productivity is due, among other factors, to occurrence of high temperatures during the summer crop season, together with the low technological level employed by producers and to shortage of improved and adapted seeds to abiotic stresses conditions, such as climatic and nutritional variations; in the latter case related mainly to N availability, among other nutritional factors (DOS SANTOS et al., 2014).

Nitrogen is the mineral macronutrient exerting the greatest influence on grain yield, as well as is the one that increases production costs (SILVA et al., 2013). However, it is fundamental for the good leaf area development, of the corn ears, and of photo-assimilates production sources (RAMBO et al., 2007), and on the increase in the content of proteins, and of the microorganisms responsible by the organic matter decomposition (MALAVOLTA, 2006).

Biological nitrogen fixation (BNF) in grasses is known since the bacterium *Beijerinckia fluminensis* was isolated from the sugarcane rhizosphere. However, it was only after the rediscovery of bacteria of the genus *Azospirillum* that the interest in the study of the nitrogen biological fixation on grasses has increased (BALDANI & BALDANI, 2005).

According to Hungria (2011), the inoculation of corn seeds with the bacterium *Azospirillum brasilense* is highly beneficial; for, besides providing nitrogen to the plants, through biological fixation, as well as by the reduction in the emission of greenhouse gases, it causes less environmental pollution resulting from the industrial production of nitrogen fertilizers.

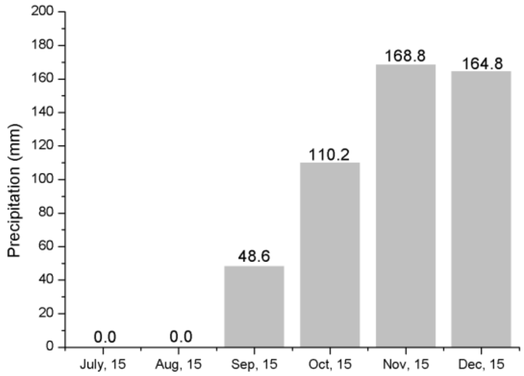
Given the above, this study aimed at assessing the effect of the bacterium *Azospirillum brasilense* inoculation, associated or not to the use of nitrogen fertilization, on the oil content of two corn genotypes grown as winter crop aiming at producing biodiesel.

MATERIAL AND METHODS

On the 2015 winter crop season, two experiments were conducted, at the Experimental Station of the Tocantins Federal University, Palmas-TO, Brazil (10º12'46''S; 48º21'37"W; 220 m altitude), one installed on July 10th and the other on August 1st, 2015. on a Red Yellow Latosol soil, with a historic of sweet potato cultivation in the last two years.

Soil analysis was performed at 0-20 cm depth, classified as Red Yellow Latosol soil, with a historic of sweet potato cultivation in the last two years. The results of the physical-chemical analysis were: OM = 19 g dm^{-3}, pH ($CaCl_2$) = 6.5, P (Mehlich) = 35 mg dm^{-3}, K = 22 mg dm^{-3}, Ca = 5.2 $cmol_c$ dm^{-3}, Mg = 2.8 $cmol_c$ dm^{-3}, CEC = 9.4, V = 86.2%, sand = 71%, clay = 23%, silt = 6% and class textural = sandy.

Monthly precipitation data (mm), and air temperature (°C), during the trials conduction period (July to December, 2015) are shown on Figure 1.



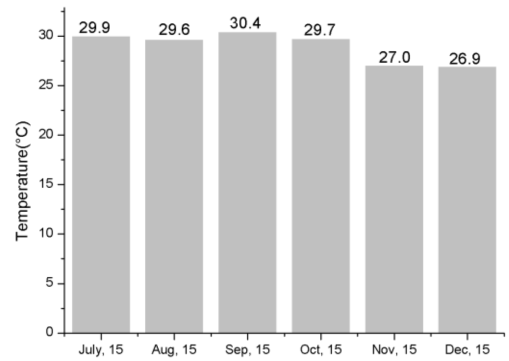


Figure 1. Monthly mean precipitations (mm) and air temperatures (ºC), which occurred from July to December during the conduction of trials on biological nitrogen fixation by the bacterium *Azospirillum brasilense*, carried out in the 2015 winter crop at the Palmas Experimental Station, State of Tocantins. Source: UFT & INMET, 2015, Palmas, TO.

Both trials were performed on a randomized complete block design with 30 treatments and three replications. Treatments were arranged on a 2x3x5 factorial scheme [2 seed inoculation processes (with and without *Azospirillum brasilense*) x 3 corn genotypes (the varieties Orion and Al Bandeirante (both from open-pollination), and the double hybrid AG-1051) x 5 different nitrogen doses (0, 50, 100, 150, and 200 kg ha^{-1})].

In treatments with the bacterium *Azospirillum brasilense*, the seeds were treated with the commercial inoculant GRAP NODa, containing the AbV5 and AbV6 strains, using a dosage of 100 ml of the inoculant to each 25 kg seeds, which were sowed immediately after treatment.

Applications of the different N doses were carried out in coverage by using Ammonium Sulphate applied in fractional doses, with half of each dose applied at the V4 vegetative stage (four fully developed leaves) and the other half applied at the V6 vegetative stage (six fully developed leaves), according to the scale of Ritchie et al. (2003). Each experimental plot was represented by two 3 m long rows, with 1 m spacing between rows, thus totaling 6 m^2.

Prior to sowing, soil was adequately plowed and harrowed, followed by opening of the furrows; which immediately after were manually fertilized with 70 kg ha^{-1} P_2O_5, in the form of single superphosphate and 48 kg ha^{-1} K_2O, in the form of potassium chloride.

Liming was not needed, since after soil analysis it was found that the pH of 6.5 was within the range considered adequate to corn crop; what agrees with Coelho & Verlengia (1973), who have reported that when pH is between 5.5 and 6.5, the maximum microbial activity in the soil occurs, and which is also very suitable to the bacterium *Azospirillum brasilense* development.

Weed control was carried out using the herbicide Atrazinax®, which was applied immediately after sowing, at the dose of 6 L ha^{-1}, as recommended by the manufacturer. However, when necessary manual weeding was also performed. Pest and disease control were not needed.

Due to lack of rainfall during trials conduction period whenever needed plots were irrigated through cannon-type sprinklers. Due to lack of rainfall during trials conduction period whenever needed plots were irrigated through cannon-type sprinklers, where it was tried to keep the soil always close to the field capacity. For the determination of the amount of water to be applied by irrigation, the water balance equation (Equation 1) was used, according to Garcia and Garcia et al. (2009), which is estimated from the crop evapotranspiration (Equation 2) and the Rega (TR) shift. The irrigation shift is the time interval, in days, between two consecutive irrigations.

IRN = ETc.TR - PE (Equation 1)

ETc = ETo. Kc (Equation 2)

Where:

IRN - Reed blade needed (mm)
ET - Evapotranspiration of the crop (mm.day-1)
TR - Watering day, days.
Pe - Effective rainfall (mm)
ETo - Reference evapotranspiration (mm.day-1)
Kc - Coefficient of maize

Harvesting was performed when grains reached physiological maturity (reproductive stage R6) by manually removing all ears from the two rows of each experimental plot, which were then duly identified. Ears of each experimental plot were threshed and grains obtained were then weighed, packed into Kraft paper bags and transferred to the Food Engineering Laboratory of Tocantins Federal University, Palmas campus, where grains were milled.

Following the Adolfo Lutz Institute (2005) recommendations, soon after milling, a sample was collected for determining the percent oil content in the grains; which was performed by the Soxhlet method of direct extraction of lipids.

The data were subjected to the F test of the analysis of variance. For the qualitative treatments (sowing dates, genotypes and inoculation processes) the means were compared by Scott-Knott test at 0.05 probability level as recommended by Borges e Ferreira (2003). Regression analysis to means of each genotype within nitrogen doses was performed through orthogonal polynomial and once the functional relationship was established between nitrogen doses and genotypes, regression equation was determined.

According to Pimentel-Gomes et al. (1985), the addition of computed values to the sources of variation results on greater F values, thus increasing probability on detecting significant differences. Therefore, aiming at obtaining a more precise experimental error, degrees of freedom for the multiple interaction: sowing times x genotypes x inoculation processes x fertilization were added to the experimental error.

To compute analysis of variance, the SISVAR 5.0 statistical program (FERREIRA, 2011) was used, and for drawing the graphs the software OriginPro Version 8.0 was employed.

RESULTS AND DISCUSSION

The analysis of variance for percent of oil content in the grains showed significant effect for all the factors analyzed individually and interactions, except to the interaction between inoculation processes x sowing times (Table 1). However, since the statistical significance of triple interactions has indicated that effects of the individual factors have not explained all variance of each individual data, the required unfolding of those data was then performed.

Table 1. Summary of the joint analysis of variance for the percentage of the oil content in the grains and of three corn genotypes, sowed with and without inoculation of *Azospirillum brasilense*, in five different nitrogen doses and two different sowing times, at Palmas Experimental Station, State of Tocantins, in the 2015 winter crop season.

SV	DF	MS Oil (%)
Sowing times	1	2.4*
Genotypes	2	48.2*
Inoculation processes	1	2.2*
Nitrogen doses	4	3.7*
Blocks x sowing times	4	0.07 ns
Sowing times x genotypes	2	4.8*
Sowing times x inoculation processes	1	0.02 ns
Sowing times x nitrogen doses	4	1.6*
Genotypes x inoculation processes	2	1.0*
Genotypes x nitrogen doses	8	4.4*
Inoculation processes x nitrogen doses	4	2.9*
Sowing times x genotypes x inoculation processes	2	7.3*
Sowing times x inoculation processes x nitrogen doses	4	2.6*
Sowing times x genotypes x nitrogen doses	8	5.4*
Genotypes x inoculation processes x nitrogen doses	8	1.8*
CV (%)		6.50
General mean		5.577

SV (Sources of Variation), DF (Degree of Freedom); MS (Middle Square)
*significant by F test at 5% of probability; ns = non-significant.

The comparative study between the means for the interactions between the treatments with and without inoculation, for each genotype at each sowing date, revealed that at the first sowing date (07/10/15), the genotypes Orion and AG-1051 showed the highest oil contents (6.2 and 4.8%, respectively) with the inoculation of the bacterium *A. brasilense*, whereas genotype Al Bandeirante, without the bacterium inoculation showed the highest value (7.0%). Nevertheless, on the second sowing date (01/08/15), genotypes Orion and Al Bandeirante presented greater oil contents (5.8 and 6.5%, respectively) when their seeds were inoculated. However, the AG-1051 genotype presented the best result when its seeds were not inoculated before seeding (Table 2).

Table 2. Mean percentages of oil content in the seeds of three different corn genotypes, inoculated (I) and non-inoculated (N/I) with the bacterium *Azospirillum brasilense*, and sown at two different seeding dates, at Palmas Experimental Station, State of Tocantins, in the 2015 winter crop season.

Genotypes	Sowing date 1 (07/10/15)		Sowing date 2 (01/08/15)	
	I	N/I	I	N/I
Orion	6.2aB1	5.6aB2	5.8bB1	5.3bB2
Al Bandeirante	6.5aA2	7.0aA1	6.5aA1	5.6bA2
AG-1051	4.8aC1	4.1bC2	4.5bC2	5.2aB1

*Means of the two sowing times, within the same inoculation processes and genotype, followed by the same lowercase letter on the line belong to same statistical group at 5% significance by Scott-Knott test; means of genotypes, within each inoculation process and in the same sowing time, followed by the same upper case letter in the column belong to same statistical group at 5% significance by Scott-Knott test; means of inoculation processes, within the same sowing time and genotype, followed by the same number on the line belong to same statistical group at 5% significance by Scott-Knott test.

In general, seed treatment with bacterium *A. brasilense* induced a greater oil content in the grains; which may be attributed to bacterial activity in the corn plant biosynthetic processes. Such effects corroborate results obtained by Didonet (1996), who has also observed that the beneficial effects attributed to the inoculation of grassy plants with bacteria of genus *Azospirillum* sp. are caused by combination of several mechanisms that together trigger different phenomena through which more complex chemical compounds are produced in those plants. In addition, according to Bashan et al. (2004), the inoculation of grasses with bacteria of this genus results in healthier and more productive plants with greater photosynthetic capacity.

Inversely, at the first sowing date, the among sowing dates comparison, within each genotype and inoculation process, has revealed a greater oil content in the grains, both to genotypes and inoculation processes.

The higher oil contents found in seeds harvested at the first sowing date are probably associated to occurrence of higher than 26 °C nocturnal temperatures (Figure 1); which have occurred during the grains filling phase (reproductive stage R2); what, according to Fancelli (2010), usually occurs between 12 and 13 weeks after germination. These results also agree to results obtained by Albrecht et al. (2008), who have likewise observed that these increases on the oil contents in legumes may be caused by biochemical disturbances in their biosynthetic processes.

In addition, during their studies Santos et al. (2016) have found that prevalence of high temperatures, as function of the increment of cellular respiration promote a well higher energetic consumption; which may cause a lower accumulation of photo assimilates and, consequently a decrease on crop yield, as well as in the oil content in grains.

Regardless inoculation processes, as well as sowing dates, among the three genotypes assessed, genotype Al Bandeirante always presented the highest oil content in grains. These results agree with results obtained by Mittelmann (2006), who stated that the differences in oil content, from one genotype to another may be explained by the fact that this parameter is quantitative and controlled by different genes; i.e., the genotype influence of the mother plant is accountable for the predominant effect in determining the oil content in grains.

According to Nehl et al. (1996), the symbiosis between the mother plant genotype and the diazotrophic bacterium inoculated is determined by the exudates quality released by the plant roots; which may have occurred more efficiently in the open-pollinated genotypes, especially for the Al Bandeirante.

To each genotype and within each nitrogen dose, the comparative study of means obtained to oil content in the grains of plants sowed with or without inoculation showed that for Orion and AG-1051 genotypes there was an increase on oil content when seeds were inoculated with *A. brasilense*, independently of applied nitrogen doses. Inversely, for genotype Al Bandeirante there was also a significant response for the increase on oil content in the grains when seeds were inoculated with the bacterium, but only when higher nitrogen doses were applied (Table 3).

According to Bashan et al. (2004), the higher oil content in the grains of corn plants that had the seeds inoculated with diazotrophic bacteria, may be attributed to the fact that inoculated plants are healthier, more productive and with greater photosynthetic capability.

Results obtained within this study also corroborate results reported by Bartchen et al. (2010) who, in studies on the effects of corn seeds inoculation, likewise have verified that inoculation of the seeds with the bacterium *A. brasilense* induced greater biological nitrogen fixation by the plants, besides providing a greater oil accumulation in the grains, in relation to plants that did not receive nitrogen fertilization in coverage.

Table 3. Mean percentages of oil content in the seeds of three corn genotypes, grown under five nitrogen doses and with the seeds inoculated (I) or non-inoculated (N/I) with the bacterium *Azospirillum brasilense*, at Palmas Experimental Station, State of Tocantins, in the 2015 winter crop season.

Nitrogen doses (kg ha^{-1})	Genotypes					
	Orion		Al Bandeirante		AG-1051	
	I	N/I	I	N/I	I	N/I
0	5.8aA1	5.2bB2	5.9aB1	6.1aB1	3.9bC2	5.4bA1
50	6.0bA1	5.7bA1	7.1aA2	7.8aA1	5.3cA1	4.7cB2
100	6.1aA1	6.0aA1	4.5bC2	5.6aC1	4.9bA1	4.9bB1
150	6.1bA1	4.9bB2	7.5aA1	6.2aB2	4.5cB1	4.6bB1
200	5.7bA1	5.4bB1	7.3aA1	6.0aB2	4.4cB1	3.5cC2

*Means of genotypes within the same inoculation process and same nitrogen dose, followed by the same lowercase letter on the line, belong to the same statistical group at 5% significance by Scott-Knott test at 5% probability; 2. Means of nitrogen doses, within each inoculation processes and for the same genotype, followed by the same upper case letter in the column belong to the same statistical group at 5% significance by Scott-Knott test at 5% probability; 3.Means of inoculation processes (I and N/I), within the same genotype and the same nitrogen dose, followed by the same number on the line belong to the same statistical group at 5% significance by Scott-Knott test at 5% probability.

Comparing the means obtained for the three genotypes assessed, within each inoculation process and within each nitrogen dose used (Table 3), it may be seen that, regardless inoculation process, the open-pollinated genotype Al Bandeirante has presented the highest oil content in the grains at almost all nitrogen doses assessed, except at the 100 kg ha^{-1} dose, at which the inoculated genotype Orion has presented the highest oil content in the grains.

The comparative study of nitrogen doses, for each genotypes and within each inoculation processes, was performed by the means test, both for genotype Orion as for genotype Al Bandeirante (both I and N/I), since there was no functional relationship for the interaction between nitrogen doses x genotypes.

For genotype Orion, it was observed that there was a higher oil content in the grains when the nitrogen doses of 50 and 100 kg ha^{-1} were applied at the sowing furrow. However, regardless inoculation for the genotype Al Bandeirante, the 50 kg ha^{-1} nitrogen dosage has provided increases on oil content in the seeds. Nevertheless, it should be emphasized that application of the nitrogen doses of 150 and 200 kg ha^{-1}, supplemented with inoculation of the seeds with the bacterium *A. brasilense* resulted on greater mean percentages of oil contents in the grains harvested.

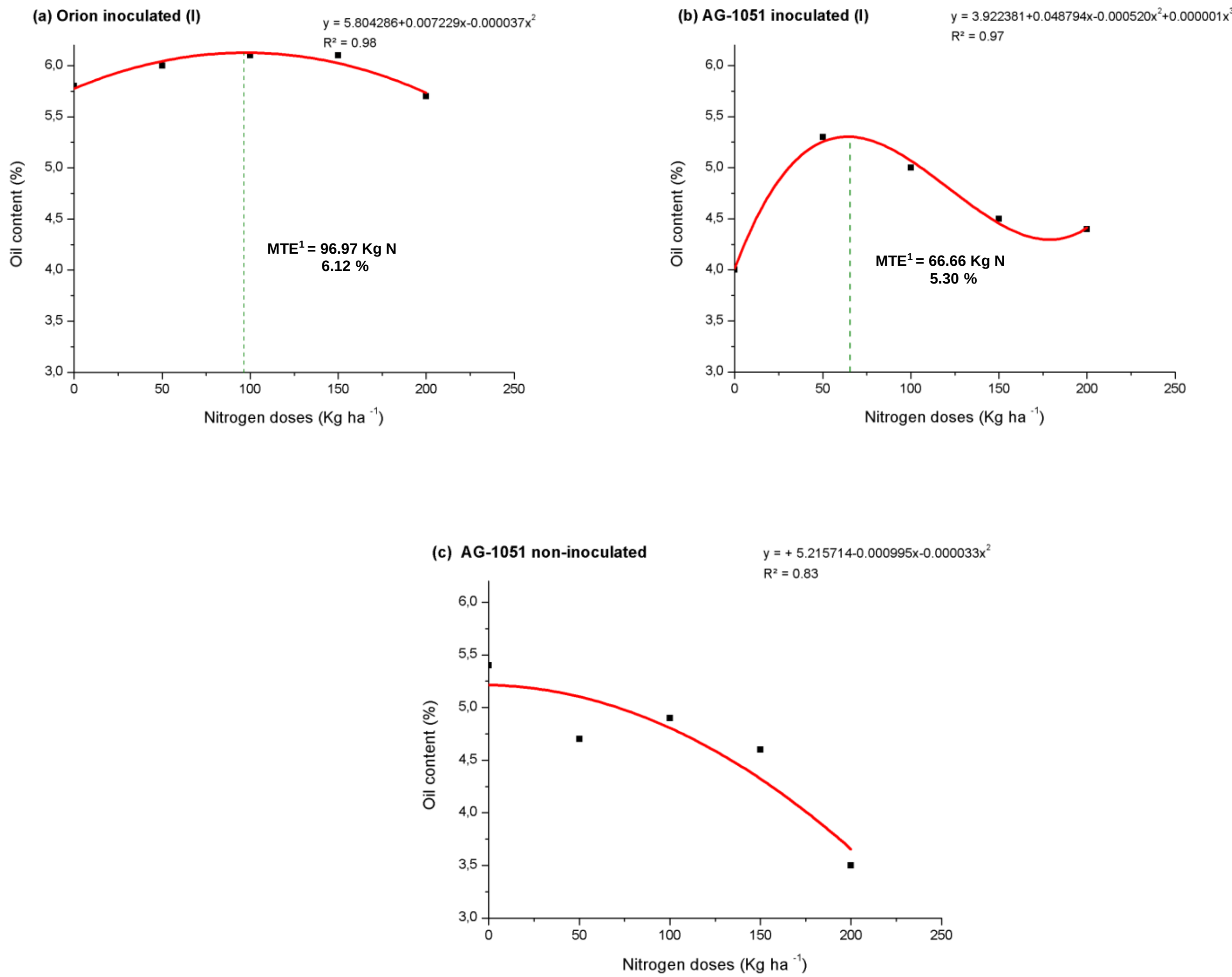
The polynomial regressions for percent oil content in the inoculated seeds (I) of genotype Orion, as well as in the non-inoculated seeds (N/I) of AG-1051, as a function of nitrogen doses, showed a quadratic model of response, as shown in Figure 3.

The Orion genotype inoculated (I) (Figure 3a) showed increases of 6.1% in the oil content until reaching the maximum technical efficiency (MTE) at the doses of approximately 96.97 kg ha^{-1}. However, as from MTE, there was reduction in the percentage of oil content in the grains. For genotype AG-1051 (Figure 3c), there was reduction with increasing nitrogen doses.

For genotype AG-1051 inoculated (I) (Figure 3b), the cubic model was the most adequate to explain the relationship between the applied nitrogen doses and percent oil content in the grains; for, through this model it was possible detecting increases on oil content until the 5.3% MTE, at dosage of approximately 66.0 kg ha^{-1} nitrogen. However, as from such dosage there was reduction on oil content in the grains.

After doses allowing obtaining the MTE to each genotype, there was always a reduction on percent of oil content, possibly due to the high doses of nitrogen applied; since, according to Perrenoud (1977) and Malavolta (1980), Carnicelli et al. (2000), the application of high doses of nitrogen, higher than those doses inducing increases until the MTE, may cause reduction on percent of oil content in the grains, due to the antagonistic effect of high doses of nitrogen in the absorption of other essential elements, mainly potassium, thereby, impairing the good development of corn plants.

In studies carried out with the soybean crop, at low latitude conditions, in the municipality of Palmas, State of Tocantins, Lima & Peluzio (2015) have also observed the positive effect of potassium on the oil content in the seeds; thus confirming the essentiality of potassium on the synthesis and transport of the oil to grains. Similar results were also obtained by Pedroso Neto & Rezende (2005), in studies performed on red-yellow Podzolic soil with clay texture in the municipality of Lavras, state of Minas Gerais, Brazil, as well as in a Dystrophic Red Latosol, with sandy-loam texture, in the municipality of Uberaba, also in the State of Minas Gerais, Brazil.



[1] Maximum efficiency; * significant at by test t at 0.05 probability.

Figure 3. Percentages of oil content in the grains of two corn genotypes, sowed with and without inoculation of the seeds with the bacterium *Azospirillum brasilense* and grown under five nitrogen doses, at Experimental Station of Palmas, in the State of Tocantins, in the 2015 winter crop season.

CONCLUSIONS

The inoculation of corn seeds with the bacterium *Azospirillum brasilense*, with or without nitrogen fertilization, promotes increases on oil content in the harvested grains.

The genotype Orion presented the greatest increase with the bacterium inoculation, as well as it was the most productive at the lowest nitrogen dosages assessed.

High temperatures favored the increase in the oil content in the grains.

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